

**METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODY-
PRODUCING CELL LINES WITH IMPROVED ANTIBODY CHARACTERISTICS**

TECHNICAL FIELD OF THE INVENTION

5 The invention is related to the area of antibody maturation and cellular production. In particular, it is related to the field of mutagenesis.

BACKGROUND OF THE INVENTION

10 The use of antibodies to block the activity of foreign and/or endogenous polypeptides provides an effective and selective strategy for treating the underlying cause of disease. In particular is the use of monoclonal antibodies (MAb) as effective therapeutics such as the FDA approved ReoPro (Glaser, V. (1996) Can ReoPro repolish tarnished monoclonal therapeutics? *Nat. Biotechnol.* 14:1216-1217), an anti-platelet MAb from Centocor; Herceptin (Weiner, L.M. (1999) Monoclonal antibody therapy of cancer. *Semin. Oncol.* 26:43-51), an anti-Her2/neu MAb from Genentech; and Synagis (Saez-Llorens, X.E., *et al.* (1998) Safety and pharmacokinetics of an intramuscular humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. *Pediat. Infect. Dis. J.* 17:787-791), an anti-respiratory syncytial virus MAb produced by Medimmune.

20 Standard methods for generating MAbs against candidate protein targets are known by those skilled in the art. Briefly, rodents such as mice or rats are injected with a purified antigen in the presence of adjuvant to generate an immune response (Shield, C.F., *et al.* (1996) A cost-effective analysis of OKT3 induction therapy in cadaveric kidney

transplantation. *Am. J. Kidney Dis.* 27:855-864). Rodents with positive immune sera are sacrificed and splenocytes are isolated. Isolated splenocytes are fused to melanomas to produce immortalized cell lines that are then screened for antibody production. Positive lines are isolated and characterized for antibody production. The direct use of rodent MAbs as human therapeutic agents were confounded by the fact that human anti-rodent antibody (HARA) responses occurred in a significant number of patients treated with the rodent-derived antibody (Khazaeli, M.B., *et al.*, (1994) Human immune response to monoclonal antibodies. *J. Immunother.* 15:42-52). In order to circumvent the problem of HARA, the grafting of the complementarity determining regions (CDRs), which are the critical motifs found within the heavy and light chain variable regions of the immunoglobulin (Ig) subunits making up the antigen binding domain, onto a human antibody backbone found these chimeric molecules are able to retain their binding activity to antigen while lacking the HARA response (Emery, S.C., and Harris, W.J. "Strategies for humanizing antibodies" In: ANTIBODY ENGINEERING C.A.K. Borrebaeck (Ed.) Oxford University Press, N.Y. 1995. pp. 159-183. A common problem that exists during the "humanization" of rodent-derived MAbs (referred to hereon as HAb) is the loss of binding affinity due to conformational changes in the 3 dimensional structure of the CDR domain upon grafting onto the human Ig backbone (U.S. Patent No. 5,530,101 to Queen *et al.*). To overcome this problem, additional HAb vectors are usually needed to be engineered by inserting or deleting additional amino acid residues within the framework region and/or within the CDR coding region itself in order to recreate high affinity HAb (U.S. Patent No. 5,530,101 to Queen *et al.*). This process is a very time consuming procedure that involves the use of expensive computer modeling programs to predict changes that may lead to a high affinity HAb. In some instances the affinity of the HAb is never restored to that of the MAb, rendering them of little therapeutic use.

Another problem that exists in antibody engineering is the generation of stable, high yielding producer cell lines that is required for manufacturing of the molecule for clinical materials. Several strategies have been adopted in standard practice by those skilled in the art to circumvent this problem. One method is the use of Chinese Hamster Ovary (CHO) cells transfected with exogenous Ig fusion genes containing the grafted human light and heavy

chains to produce whole antibodies or single chain antibodies, which are a chimeric molecule containing both light and heavy chains that form an antigen-binding polypeptide (Reff, M.E. (1993) High-level production of recombinant immunoglobulins in mammalian cells. *Curr. Opin. Biotechnol.* 4:573-576). Another method employs the use of human lymphocytes derived from transgenic mice containing a human grafted immune system or transgenic mice containing a human Ig gene repertoire. Yet another method employs the use of monkeys to produce primate MAbs, which have been reported to lack a human anti-monkey response (Neuberger, M., and Gruggermann, M. (1997) Monoclonal antibodies. Mice perform a human repertoire. *Nature* 386:25-26). In all cases, the generation of a cell line that is capable of generating sufficient amounts of high affinity antibody poses a major limitation for producing sufficient materials for clinical studies. Because of these limitations, the utility of other recombinant systems such as plants are currently being explored as systems that will lead to the stable, high-level production of humanized antibodies (Fiedler, U., and Conrad, U. (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technology* 13:1090-1093).

A method for generating diverse antibody sequences within the variable domain that results in HAbs and MAbs with high binding affinities to antigens would be useful for the creation of more potent therapeutic and diagnostic reagents respectively. Moreover, the generation of randomly altered nucleotide and polypeptide residues throughout an entire antibody molecule will result in new reagents that are less antigenic and/or have beneficial pharmacokinetic properties. The invention described herein is directed to the use of random genetic mutation throughout an antibody structure *in vivo* by blocking the endogenous mismatch repair (MMR) activity of a host cell producing immunoglobulins that encode biochemically active antibodies. The invention also relates to methods for repeated *in vivo* genetic alterations and selection for antibodies with enhanced binding and pharmacokinetic profiles.

In addition, the ability to develop genetically altered host cells that are capable of secreting increased amounts of antibody will also provide a valuable method for creating cell hosts for product development. The invention described herein is directed to the creation of genetically altered cell hosts with increased antibody production via the blockade of MMR.

The invention facilitates the generation of high affinity antibodies and the production of cell lines with elevated levels of antibody production. Other advantages of the present invention are described in the examples and figures described herein.

5 SUMMARY OF THE INVENTION

The invention provides methods for generating genetically altered antibodies (including single chain molecules) and antibody producing cell hosts *in vitro* and *in vivo*, whereby the antibody possess a desired biochemical property(s), such as, but not limited to, increased antigen binding, increased gene expression, and/or enhanced extracellular secretion
10 by the cell host. One method for identifying antibodies with increased binding activity or cells with increased antibody production is through the screening of MMR defective antibody producing cell clones that produce molecules with enhanced binding properties or clones that have been genetically altered to produce enhanced amounts of antibody product.

The antibody producing cells suitable for use in the invention include, but are not
15 limited to rodent, primate, or human hybridomas or lymphoblastoids; mammalian cells transfected and expressing exogenous Ig subunits or chimeric single chain molecules; plant cells, yeast or bacteria transfected and expressing exogenous Ig subunits or chimeric single chain molecules.

Thus, the invention provides methods for making hypermutable antibody-producing cells by introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into cells that are capable of producing antibodies. The cells that are capable of producing antibodies include cells that naturally produce antibodies, and cells that are engineered to produce antibodies through the introduction of immunoglobulin encoding sequences. Conveniently, the introduction of polynucleotide sequences into cells is accomplished by transfection.

The invention also provides methods of making hypermutable antibody producing cells by introducing a dominant negative mismatch repair (MMR) gene such as *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2* into cells that are capable of producing antibodies. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134,

or a thymidine at nucleotide 424 of wild-type *PMS2*). The invention also provides methods in which mismatch repair gene activity is suppressed. This may be accomplished, for example, using antisense molecules directed against the mismatch repair gene or transcripts.

Other embodiments of the invention provide methods for making a hypermutable antibody producing cells by introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into fertilized eggs of animals. These methods may also include subsequently implanting the eggs into pseudo-pregnant females whereby the fertilized eggs develop into a mature transgenic animal. The mismatch repair genes may include, for example, *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2*. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*).

The invention further provides homogeneous compositions of cultured, hypermutable, mammalian cells that are capable of producing antibodies and contain a dominant negative allele of a mismatch repair gene. The mismatch repair genes may include, for example, *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2*. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*). The cells of the culture may contain *PMS2*, (preferably human *PMS2*), *MLH1*, or *PMS1*; or express a human *mutL* homolog, or the first 133 amino acids of hPMS2.

The invention further provides methods for generating a mutation in an immunoglobulin gene of interest by culturing an immunoglobulin producing cell selected for an immunoglobulin of interest wherein the cell contains a dominant negative allele of a mismatch repair gene. The properties of the immunoglobulin produced from the cells can be assayed to ascertain whether the immunoglobulin gene harbors a mutation. The assay may be directed to analyzing a polynucleotide encoding the immunoglobulin, or may be directed to the immunoglobulin polypeptide itself.

The invention also provides methods for generating a mutation in a gene affecting antibody production in an antibody-producing cell by culturing the cell expressing a dominant negative allele of a mismatch repair gene, and testing the cell to determine whether the cell

harbors mutations within the gene of interest, such that a new biochemical feature (*e.g.*, over-expression and/or secretion of immunoglobulin products) is generated. The testing may include analysis of the steady state expression of the immunoglobulin gene of interest, and/or analysis of the amount of secreted protein encoded by the immunoglobulin gene of interest. The invention also embraces prokaryotic and eukaryotic transgenic cells made by this process, including cells from rodents, non-human primates and humans.

Other aspects of the invention encompass methods of reversibly altering the hypermutability of an antibody producing cell, in which an inducible vector containing a dominant negative allele of a mismatch repair gene operably linked to an inducible promoter is introduced into an antibody-producing cell. The cell is treated with an inducing agent to express the dominant negative mismatch repair gene (which can be *PMS2* (preferably human *PMS2*), *MLH1*, or *PMS1*). Alternatively, the cell may be induced to express a human *mutL* homolog or the first 133 amino acids of hPMS2. In another embodiment, the cells may be rendered capable of producing antibodies by co-transfecting a preselected immunoglobulin gene of interest. The immunoglobulin genes of the hypermutable cells, or the proteins produced by these methods may be analyzed for desired properties, and induction may be stopped such that the genetic stability of the host cell is restored.

The invention also embraces methods of producing genetically altered antibodies by transfecting a polynucleotide encoding an immunoglobulin protein into a cell containing a dominant negative mismatch repair gene (either naturally or in which the dominant negative mismatch repair gene was introduced into the cell), culturing the cell to allow the immunoglobulin gene to become mutated and produce a mutant immunoglobulin, screening for a desirable property of said mutant immunoglobulin protein, isolating the polynucleotide molecule encoding the selected mutant immunoglobulin possessing the desired property, and transfecting said mutant polynucleotide into a genetically stable cell, such that the mutant antibody is consistently produced without further genetic alteration. The dominant negative mismatch repair gene may be *PMS2* (preferably human *PMS2*), *MLH1*, or *PMS1*. Alternatively, the cell may express a human *mutL* homolog or the first 133 amino acids of hPMS2.

The invention further provides methods for generating genetically altered cell lines that express enhanced amounts of an antigen binding polypeptide. These antigen-binding polypeptides may be, for example, immunoglobulins. The methods of the invention also include methods for generating genetically altered cell lines that secrete enhanced amounts of an antigen binding polypeptide. The cell lines are rendered hypermutable by dominant negative mismatch repair genes that provide an enhanced rate of genetic hypermutation in a cell producing antigen-binding polypeptides such as antibodies. Such cells include, but are not limited to hybridomas. Expression of enhanced amounts of antigen binding polypeptides may be through enhanced transcription or translation of the polynucleotides encoding the antigen binding polypeptides, or through the enhanced secretion of the antigen binding polypeptides, for example.

Methods are also provided for creating genetically altered antibodies *in vivo* by blocking the MMR activity of the cell host, or by transfecting genes encoding for immunoglobulin in a MMR defective cell host.

Antibodies with increased binding properties to an antigen due to genetic changes within the variable domain are provided in methods of the invention that block endogenous MMR of the cell host. Antibodies with increased binding properties to an antigen due to genetic changes within the CDR regions within the light and/or heavy chains are also provided in methods of the invention that block endogenous MMR of the cell host.

The invention provides methods of creating genetically altered antibodies in MMR defective Ab producer cell lines with enhanced pharmacokinetic properties in host organisms including but not limited to rodents, primates, and man.

These and other aspects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention, a method for making an antibody producing cell line hypermutable is provided. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into an antibody-producing cell. The cell becomes hypermutable as a result of the introduction of the gene.

In another embodiment of the invention, a method is provided for introducing a mutation into an endogenous gene encoding for an immunoglobulin polypeptide or a single chain antibody. A polynucleotide encoding a dominant negative allele of a MMR gene is

introduced into a cell. The cell becomes hypermutable as a result of the introduction and expression of the MMR gene allele. The cell further comprises an immunoglobulin gene of interest. The cell is grown and tested to determine whether the gene encoding for an immunoglobulin or a single chain antibody of interest harbors a mutation. In another aspect of the invention, the gene encoding the mutated immunoglobulin polypeptide or single chain antibody may be isolated and expressed in a genetically stable cell. In a preferred embodiment, the mutated antibody is screened for at least one desirable property such as, but not limited to, enhanced binding characteristics.

In another embodiment of the invention, a gene or set of genes encoding for Ig light and heavy chains or a combination therein are introduced into a mammalian cell host that is MMR defective. The cell is grown, and clones are analyzed for antibodies with enhanced binding characteristics.

In another embodiment of the invention, a method will be provided for producing new phenotypes of a cell. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene. The cell is grown. The cell is tested for the expression of new phenotypes where the phenotype is enhanced secretion of a polypeptide.

These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in cells and animals as well as providing cells and animals harboring potentially useful mutations for the large-scale production of high affinity antibodies with beneficial pharmacokinetic profiles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Hybridoma cells stably expressing PMS2 and PMS134 MMR genes.

Shown is steady state mRNA expression of MMR genes transfected into a murine hybridoma cell line. Stable expression was found after 3 months of continuous growth. The (-) lanes represent negative controls where no reverse transcriptase was added, and the (+) lanes represent samples reverse transcribed and PCR amplified for the MMR genes and an internal housekeeping gene as a control.

Figure 2. Creation of genetically hypermutable hybridoma cells. Dominant negative

MMR gene alleles were expressed in cells expressing a MMR-sensitive reporter gene. Dominant negative alleles such as PMS134 and the expression of MMR genes from other species results in antibody producer cells with a hypermutable phenotype that can be used to produce genetically altered immunoglobulin genes with enhanced biochemical features as well as lines with increased Ig expression and/or secretion. Values shown represent the amount of converted CPRG substrate which is reflective of the amount of function β -galactosidase contained within the cell from genetic alterations within the pCAR-OF reporter gene. Higher amounts of β -galactosidase activity reflect a higher mutation rate due to defective MMR.

Figure 3. Screening method for identifying antibody-producing cells containing antibodies with increased binding activity and/or increased expression/secretion

Figure 4. Generation of a genetically altered antibody with an increased binding activity. Shown are ELISA values from 96-well plates, screened for antibodies specific to hIgE. Two clones with a high binding value were found in HB134 cultures.

Figure 5. Sequence alteration within variable chain of an antibody (a mutation within the light chain variable region in MMR-defective HB134 antibody producer cells). Arrows indicate the nucleotide at which a mutation occurred in a subset of cells from a clone derived from HB134 cells. Panel A: The change results in a Thr to Ser change within the light chain variable region. The coding sequence is in the antisense direction. Panel B: The change results in a Pro to His change within the light chain variable region.

Figure 6. Generation of MMR-defective clones with enhanced steady state Ig protein levels. A Western blot of heavy chain immunoglobulins from HB134 clones with high levels of MAb (>500ngs/ml) within the conditioned medium shows that a subset of clones express higher steady state levels of immunoglobulins (Ig). The H36 cell line was used as a control to measure steady state levels in the parental strain. Lane 1: fibroblast cells (negative control); Lane 2: H36 cell; Lane 3: HB134 clone with elevated MAb levels; Lane 4: HB134 clone with elevated MAb levels; Lane 5: HB134 clone with elevated MAb levels.

Methods have been discovered for developing hypermutable antibody-producing cells by taking advantage of the conserved mismatch repair (MMR) process of host cells.

Dominant negative alleles of such genes, when introduced into cells or transgenic animals,

increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable. Hypermutable cells or animals can then be utilized to develop new mutations in a gene of interest. Blocking MMR in antibody-producing cells such as but not limited to: hybridomas; mammalian cells transfected with genes encoding for Ig light and heavy chains; mammalian cells transfected with genes encoding for single chain antibodies; eukaryotic cells transfected with Ig genes, can enhance the rate of mutation within these cells leading to clones that have enhanced antibody production and/or cells containing genetically altered antibodies with enhanced biochemical properties such as increased antigen binding. The process of MMR, also called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells. A MMR gene is a gene that encodes for one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a MMR complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base, which is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication.

Dominant negative alleles cause a MMR defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a MMR gene is the human gene *hPMS2-134*, which carries a truncating mutation at codon 134 (SEQ ID NO:15). The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations, which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any allele which produces such effect can be used in this invention. Dominant negative alleles of a MMR gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms. Such alleles can be identified by screening cells for defective MMR activity. Cells from animals or humans with cancer can be screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic

DNA, cDNA, or mRNA from any cell encoding a MMR protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a MMR gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other MMR genes. Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or animals can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

A cell or an animal into which a dominant negative allele of a mismatch repair gene has been introduced will become hypermutable. This means that the spontaneous mutation rate of such cells or animals is elevated compared to cells or animals without such alleles. The degree of elevation of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or animal. The use of chemical mutagens such as but limited to methane sulfonate, dimethyl sulfonate, O6-methyl benzadine, MNU, ENU, etc. can be used in MMR defective cells to increase the rates an additional 10 to 100 fold that of the MMR deficiency itself.

According to one aspect of the invention, a polynucleotide encoding for a dominant negative form of a MMR protein is introduced into a cell. The gene can be any dominant negative allele encoding a protein, which is part of a MMR complex, for example, *PMS2*, *PMS1*, *MLH1*, or *MSH2*. The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide.

The polynucleotide can be cloned into an expression vector containing a constitutively active promoter segment (such as but not limited to CMV, SV40, Elongation Factor or LTR sequences) or to inducible promoter sequences such as the steroid inducible pIND vector (Invitrogen), where the expression of the dominant negative MMR gene can be regulated. The polynucleotide can be introduced into the cell by transfection.

According to another aspect of the invention, an immunoglobulin (Ig) gene, a set of Ig genes or a chimeric gene containing whole or parts of an Ig gene can be transfected into MMR deficient cell hosts, the cell is grown and screened for clones containing genetically altered Ig genes with new biochemical features. MMR defective cells may be of human,

primates, mammals, rodent, plant, yeast or of the prokaryotic kingdom. The mutated gene encoding the Ig with new biochemical features may be isolated from the respective clones and introduced into genetically stable cells (*i.e.*, cells with normal MMR) to provide clones that consistently produce Ig with the new biochemical features. The method of isolating the

5 Ig gene encoding Ig with new biochemical features may be any method known in the art. Introduction of the isolated polynucleotide encoding the Ig with new biochemical features may also be performed using any method known in the art, including, but not limited to transfection of an expression vector containing the polynucleotide encoding the Ig with new biochemical features. As an alternative to transfecting an Ig gene, a set of Ig genes or a
10 chimeric gene containing whole or parts of an Ig gene into an MMR deficient host cell, such Ig genes may be transfected simultaneously with a gene encoding a dominant negative mismatch repair gene into a genetically stable cell to render the cell hypermutable.

Transfection is any process whereby a polynucleotide is introduced into a cell. The process of transfection can be carried out in a living animal, *e.g.*, using a vector for gene
15 therapy, or it can be carried out *in vitro*, *e.g.*, using a suspension of one or more isolated cells in culture. The cell can be any type of eukaryotic cell, including, for example, cells isolated from humans or other primates, mammals or other vertebrates, invertebrates, and single celled organisms such as protozoa, yeast, or bacteria.

In general, transfection will be carried out using a suspension of cells, or a single cell,
20 but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the
25 use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the MMR gene, the cell can be grown and reproduced in culture. If the transfection is stable, such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

An isolated cell is a cell obtained from a tissue of humans or animals by mechanically
30 separating out individual cells and transferring them to a suitable cell culture medium, either

with or without pretreatment of the tissue with enzymes, *e.g.*, collagenase or trypsin. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a eukaryotic organism in the form of a primary cell culture or an immortalized cell line, or
5 may be derived from suspensions of single-celled organisms.

A polynucleotide encoding for a dominant negative form of a MMR protein can be introduced into the genome of an animal by producing a transgenic animal. The animal can be any species for which suitable techniques are available to produce transgenic animals. For example, transgenic animals can be prepared from domestic livestock, *e.g.*, bovine, swine,
10 sheep, goats, horses, etc.; from animals used for the production of recombinant proteins, *e.g.*, bovine, swine, or goats that express a recombinant polypeptide in their milk; or experimental animals for research or product testing, *e.g.*, mice, rats, guinea pigs, hamsters, rabbits, etc. Cell lines that are determined to be MMR defective can then be used as a source for producing genetically altered immunoglobulin genes *in vitro* by introducing whole, intact
15 immunoglobulin genes and/or chimeric genes encoding for single chain antibodies into MMR defective cells from any tissue of the MMR defective animal.

Once a transfected cell line or a colony of transgenic animals has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest can be any gene naturally possessed by the cell line or transgenic animal or introduced into
20 the cell line or transgenic animal. An advantage of using such cells or animals to induce mutations is that the cell or animal need not be exposed to mutagenic chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers. However, chemical mutagens may be used in combination with MMR deficiency, which renders such mutagens less toxic due to an undetermined mechanism. Hypermutable
25 animals can then be bred and selected for those producing genetically variable B-cells that may be isolated and cloned to identify new cell lines that are useful for producing genetically variable cells. Once a new trait is identified, the dominant negative MMR gene allele can be removed by directly knocking out the allele by technologies used by those skilled in the art or by breeding to mates lacking the dominant negative allele to select for offspring with a
30 desired trait and a stable genome. Another alternative is to use a CRE-LOX expression

system, whereby the dominant negative allele is spliced from the animal genome once an animal containing a genetically diverse immunoglobulin profile has been established. Yet another alternative is the use of inducible vectors such as the steroid induced pIND (Invitrogen) or pMAM (Clonetech) vectors which express exogenous genes in the presence of corticosteroids.

Mutations can be detected by analyzing for alterations in the genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by screening for the production of antibody titers. A mutant polypeptide can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or animal associated with the function of the gene of interest, such as but not limited to Ig secretion.

Examples of mismatch repair proteins and nucleic acid sequences include the following:

PMS2 (mouse) (SEQ ID NO:5)

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MEQTEGVSTE CAKAIKPIDG KSVHQICSGQ VILSLSTAVK ELIENSVDAG ATTIDLRLEK 60
YGVDLIEVSD NGCGVEEENF EELALKHHTS KIQEEADLTQ VETFGFGEA LSSLCALSLV 120
TISTCHGSAS VGTRLVEDEN GKITQKTPYP PPKGTTVSQV HLFYTLVPY KEFQENIKKE 180
YSKMWQVLQA YCIISAGVEV SOTNQLGQGH FHAVVCTSGT SGMKENIGSV FGQKQLQSLI 240
PFVQLPFSDA VCEEYGLSTS GRHKTFSTFR ASFHSAATAP GGVQQTGSFS SSIRGPVTQQ 300
PSSLSLMPFY HMYNKHQYFF VVINVSVDSE CVDINVTPEK PQIILQEEKL LLAVLKTSLI 360
GMFLSDANKL NVNQQLLDV EGNILVKLHTA ELEKPVFGKQ INSPSLKSTA DEKRVASISR 420
LREAFSLHPT KEIKSRGPET AELTRSFPSK KRGVLSSYPS IVISYFGLFG SQDKLVSPDT 480
SPGDCMDREK IFKDSGLSST SAGSEEEFST FEVASSFSSD YNVSSLEDFP SQETINCGDL 540
DCRFPGTGQS LKPEDHGYQC KALPIARLSP TNAKRFKTEF FPSNVNLSQF LPPGPQSTSA 600
EVDVAIKMNK RIVLLEFSLH SLAKRMKQLQ HLKAQNKHEL SYPRFFAKIC FGENQAADDE 660
LRKEISKSMF AEMETLGQFN LGFLVTKLKE DLFLVDQHAA DEKYNFEMLE QHTVLQAQRL 720
ITPQTLNLTA VNEAVLIENL EIFRKNIGDF VIDEADPVTE FAKLISLPTS KNWTFGPQDI 780
DELIFMLSDS PGVMCRPSRV RQMFASRACK KSMIGTALN ASEMKKKLTH MGEMDHPWNC 840
PHGRPTMRHV ANLDVISQI 859

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PMS2 (mouse cDNA) (SEQ ID NO:6)

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gaattccgggt gaaggttcctg aagaatttcc agatttcctga gtatttcctg agggagacaga 60
taacctgctg ccaggttaacg atggtgtata tgcacacagaa atgggtgttc ctggagacgg 120
gtctctctccc gagagcgcca ccggaactct ccggcggtga ctgtgactgg aggagtcctg 180
catccatgga gcaaacggaa ggcttgagta cagaatgtgc taaggccatc aagcctattg 240
atgggaagtc agtccatcaa attgttctg ggcaggtgat actcagttta agcacggctg 300
tgaaggagtt gatagaaaat agttagatg ctggtgctac tactattgat ctaaggctta 360
aagaactatgg ggtggacctc attgaagttt cagacaatgg atgtggggta gaagaagaaa 420

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aatttgaagg tctagctctg aaacatcaca catctaagat tcaagagttt goocacotca 480
 cgcagggttg aaatttoggc tttcgggggg aagctctgag ctctctgtgt goactaactc 540
 atgtcactat atctacctgc caaggtcttg caagcgttgg gactcgactg gtgtttgacc 600
 ataatgggaa aatcaccagc aaaaatccct acccccgacc taaaggaacc acaatbaytg 660
 5 tgcagcactt attttatata ctacccgtgc gtacaaaaga gtttcagagg aacattakaa 720
 appagtattc aaaaatgggt caggtcttac aggcgtactg tctcatctca gcagggttcc 780
 gtgtaaactg caataatcag ctgggacagg ggaagcggca cgtgtgtgtg tgcacaaagc 840
 gtaagtctgg catqaaggaa aatctccggt ctgtgttttg ccagaagcag tctgaaagcc 900
 tttattcttc ttttccagtg cccctcagtg aactgtgtt tgaagagtao ggortgagca 960
 10 ctccaggaag ctacaaaacc ttttccaggt ttgggtcttc atttcacagt ggaagcaggy 1020
 cttccgggag apttcaacag acaggpagit tttcttcatc aatcagaggc cctctgaccc 1080
 aytaaaagtc tctaaagcttg tcaatgaggt tttatccatc gtataacccg catcaytaac 1140
 catcttgctg cttttaaagtc tcccttgact caaatctggt gcatattaat gtaactccag 1200
 ataaaaagca aattctacta caaagaagaa agtattgtct ggcgtcttta agaatccct 1260
 15 tpataggaat gtttgacagt gatcacaaca agtttaalgc caaccagcag ccactgctag 1320
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PMS2 (human) (SEQ ID NO:7)

MEKAESSSTE FAKALKFIDR KSVHQCICSGQ VVLSLSTAVK ELVENSLLAG ATHIFIKLKD 60
 VGVDLIEVSD NGCGVEEENF EGLTLKHHTS HQCEYALLTQ VETFGFFGEA LSSLGALSTV 120
 50 TLSTCHASAK VETHLMFDHN GHILQKTFYF PFGGTIVSVQ QLFSTLEVPF KEFQENIKKE 180
 YAKMVQVLHA PTHISAGIEV STTNQLGQCK PQPVVCTGGS PSIKENIGSV FQKQLQSLI 240
 EFVQLFESUS VCEFYGLSCS DALHNLFIYS GFISQCTHGV PSSTTFQFF FLMFPCDFA 300
 KVCFLNVEVY HMTNFEQYTF VVINISNDSB CVDINVTOK EQILLQFEKL TLAVLKTSLI 360
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 55 EFATSLFHTT ENNPESEKTP HFRSPLEQK FGMLESSTSG AITCKGVLP QREAVSSSHG 480
 EEDFTEAEV EKDSCGGSTV VDSGFSIFD TSGDSSFTY ASSIGLFSSQ EAVLQENAP 540
 ETDGSEFND THNQEOTEC KFFVLPQTH LATENTREK KEELISSLI QELINTHLM 600
 SASIVENAVK INKKNVPLIF SMESLAKLIK QLEHEAQQSE EQONYAKFA KICFGENMA 660
 60 EDELKKEISK TMFAEMRIG QNLGFIITK LNEQIFTVOQ HATLEKNYFE MLQHTVVLQ 720
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 QOVDELIFML SDSPGVMDRP SVKQMFASR ATRKSYMIGT ALNTSEMHL ITHGEMDHP 840
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PMS2 (human cDNA) (SEQ ID NO:8)

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PMS1 (human) (SEQ ID NO:9)

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 YVLDGSGRIL SQFESHLCQG TTVTALFIFK NLEVEKQFYS TAPKCFDEIF KICQLLMSFG 180
 ILKEFLFIVE VHKAVIWQK SFVSDHKMAL MSVLGTAVMN NMESFQYHSE ESQIYLSGFL 240
 EPCDAHSFT SLSTPERSFI FINSFPVRQK IILMLIFHHY NLRCLKESTE IYFVFLKID 300
 VPTAIVLVNL TFCFSQVLLQ NFBSVLIALE NLMTTCYGFEL PSTNSYENNK TIVSAADIVL 360
 SKTAETDVLF NKVESSGKNY SNVETSVIFF QNMENNESG KNTLEPLNHQ LSIGFPGYGH 420
 CSSEISNLIK NTENAPQDIS MSNYSWENSQ TEYSKTCFIS SVFHTSPFNG NKDHIESGE 480
 60 NEEFAGLENS SEISADEWSR GNILKNSVGE NIEPVHILVP EKSILPKVSN NNYPIFEQMN 540
 LNELSONKKS NVILNKSQKV TAYDLLSNFV IKKPMASASL FVQDHFQFL IENPKTSLED 600

ATLQIEELWK TLSEEEYLFY EEKATHDLER YNSQMKAIE QESQMSLKG RFWIKPTSAW 660
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 5 CLPFGVGAUL KEILNAILNE NAEVNYECFF FFWISYLEGE AVRLSQQPM YLSPHEDIQDI 910
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PMS1 (human) (SEQ ID NO:10)

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MSH2 (human) (SEQ ID NO:11)

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  NDQFSNLEAL LIIGSPKECV LIGGETAGSM GKLPJIDGFQ GILITFEKFA IFTTHDIYID 241
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10 PLDFAAVFAL NLFVGSVEDT TGSLSLAALL NPGHIFGSR LTNWVWQPL MDGNFIHEAL 361
  NLVEAFVETA ELFTLQEDL LARFRLNHL AKYFQGLAN LQDNYHLYQG INLFNVVQA 421
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  TVLIQKNGVH FINSKLTSLN ERYTHNTRY EEAQDAIWE INNISSEYVE IMITINIEIA 601
15 QILAVVSAFH VSNQAPVEYV FIALEFPGZF FILLPASHHA QWNGTEIAF IPNLVPEKI 661
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MSH2 (human cDNA) (SEQ ID NO:12)

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| | atatttaacc | tttttccata | gtgttcaactg | tcagtgcaca | tggtgtatca | aacttaataag | 3000 |
| | atattttagta | atattttact | ttgaggagat | tttcaaaagt | ttctatcttg | aaaaaatgaga | 3060 |
| 15 | gtgttaactg | aggagtgttt | gcaatgaca | taggcaataa | taagtgtgtt | gttgaatttt | 3120 |
| | ataaataaaa | tcattgtagtt | tgtgg | | | | 3180 |

MLH1 (human) (SEQ ID NO:13)

| | | | | | | | |
|----|-------------|------------|------------|------------|------------|------------|-----|
| | MSEVAVSVER | IDETVNFIA | ASEVIGFPAN | AIFEMENCL | DAFSTSLQVI | VFEGLKLIQ | 60 |
| 20 | IQNGTGIFH | FCLLVCEFF | TTSLHSFED | LASISTYGR | GFALASISHV | AEVITITKTA | 120 |
| | EGHCATPASY | SDGLKAPFK | ETAGNQCTOI | TVFELFYHIA | TFEKALFNPS | EFYGRILEVV | 180 |
| | QPSVHNADI | SFSVKKQGEI | VALVFILPNA | SIVNIESIF | GNAVSEELIE | IGCEKTLAF | 240 |
| | PMNGYISNAN | YSVKKCIIL | FINHFLVEST | SLFKAITVY | AAYLEKNTHP | FLYLSLEISP | 300 |
| | QNVIVNVHPT | FHEVHFLREE | SILEPVQQHI | ESKILGSNS | EMYFIQTLLF | GLAGHSGEMV | 360 |
| 25 | FSTTSLTSSS | TSOSSSEVYA | HNVFTDSFE | QHLIAFLQFL | SFHLSSLPQA | IVTEFTDIS | 420 |
| | SCFAPQDEE | MLELPALAV | AAKNSLEED | ITFGTSEMSE | KFGTSSNPF | KRHEEDSDVE | 480 |
| | NVEIISFKEM | TAACITFPRI | INLTSVLSIQ | EEINEQCHEV | LFEMLRHESF | VGVNHPQWAL | 540 |
| | AQHQTCLYLL | NTTELSEELF | YGLIYDFAN | FGVLFLEPFA | FLFLAMLAL | ESPEGWTEE | 600 |
| | DGPREGLAAY | IVEFLKKKAE | MLADYFSLET | DEEGLNLGLP | LLIINTVIFL | EGLPIFILEL | 660 |
| 30 | ATEVINWDEEK | ECFESLSKEC | AMFYSIFKQT | ISEESTLSGQ | QSEVFGSIPN | SWKWTVEHIV | 720 |
| | YKALFSEILP | PERFTEDGHI | LQIANLFLPY | KVFEFC | | | 780 |

MLH1 (human) (SEQ ID NO:14)

| | | | | | | | |
|----|-------------|-------------|-------------|-------------|------------|-------------|------|
| | cttctgtctt | ctggcgccaa | aatgttgttt | gtggcagggg | ttattcggcg | gttggacgag | 60 |
| 35 | acagtggtga | acgcgctcgc | ggcgggggaa | gttatccagc | ggcaggttaa | tgctatcaaa | 120 |
| | gagatgattg | agaaatgttt | agatgcaaaa | tcacaaagta | ttcaggtgat | tgtaaaagag | 180 |
| | ggagccttga | agttgattca | gacccaaagc | aatggccacg | ggatcaggaa | agaagatctg | 240 |
| | gataatgtat | gtgaaagggt | caatactagt | aaactgcagt | cccttgagga | tttagccagt | 300 |
| | attctctact | atggtcttgc | aggtgaggtt | ttggccagca | taagcctagt | ggctcaggtt | 360 |
| 40 | actattacaa | cgaaaacagc | tgatggaaag | tgtgtatada | gagcgaagta | ctcagatgga | 420 |
| | aaactggaaag | ccctctctaa | accatgtctt | ggcaatcaag | ggacccagat | caaggtggag | 480 |
| | gactcttttt | acaaacatagc | ccagaggaga | aaagctttaa | aaaatccaa | tgaaagaatat | 540 |
| | gggaaaaatt | tggaaattgt | tggcaggtat | tcagtaacaa | atgcaggcat | taattttctca | 600 |
| | gttaaaaaaac | aaggagagac | agtagctgat | gttaggacac | taacaaatgc | ctcaacccgtg | 660 |
| 45 | gacaatattc | gtcccatctt | tggaaatgct | gttagtcag | aatgcataga | aattggatgt | 720 |
| | gaggataaaa | ccctagcctt | caaaatgaat | ggttacatat | ccaatgcaaa | ctactcagtg | 780 |
| | aaagaagtga | ctctcttaact | cttcatcaac | catcgtcttg | tagaatcaac | ttccttgaga | 840 |
| | aaagccatag | aaacagtgca | tgagccttat | ttggccaaaa | acacacaccc | attcctgtac | 900 |
| | ctcagtttag | aaatcagttc | ccagaattgtg | gatgttaattg | tgcaacccac | aaagpatgaa | 960 |
| 50 | gttcactctc | tgcacgagga | gagcctcctg | gagcgggtgc | agcagacat | cgagagcaag | 1020 |
| | ctccggggt | ccaattcttc | caggatgac | ttcaccaga | ctctgttacc | aggatttgtt | 1080 |
| | ggcctctctg | gggagatggt | taaatccaca | acaaatctga | ctctgtcttc | taactcttga | 1140 |
| | agtagtata | aggtctatgc | ccaccagatg | gttcgtacag | attcccgga | acagaagctt | 1200 |
| | gatgatttct | tgcagcctct | gagcaaaccc | ctgtccagtc | agccccagga | cattgtccaca | 1260 |
| 55 | gaggataaga | cagatatttc | catggccagg | gctagccagc | aagatgagga | gatccttgaa | 1320 |
| | ctccacagccc | ctgctgaagt | ggatgcacaa | aatcagagct | tggaggggga | tacaacaaag | 1380 |
| | gggacttcag | aaatgtccaga | gaagagagga | ctcacttcca | gcaaccccag | aaagagacat | 1440 |
| | cggaagatt | ctgatgtgga | aatggtggaa | gatgattccc | gaaaggaaat | gactgcagct | 1500 |

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5  tgtacccccccc ggagaaggat cattaacotc actagtgttt tgagtctcca ggaagaaatt 1160
aatgagcagg gacatgaggt tctcggggag atgtttgcata accactcctt cgtgggctgt 1170
gtgaatcctc agtgggocctt ggcacagcat caaaaccaagt tatacctttt caacaccacc 1180
aaggttagtg aagaactgtt ctaccagata ctcatctatg attttgccaa ttttggtgtt 1190
ctcaggttat cggagccagc accgtctctt gaccttgcca tgcttgccct agatagtcca 1200
gagagtgggt ggcacagagga agatggctcc aaagaaggac ttgctgaata cattgttgag 1210
tttctgaaga agaaggctga gatgcttgca gactatttct ctttggaat tgatgaggaa 1220
gggaacctga ttggattacc ccttctgatt gacaaactatg tgcccccctt ggagggaactg 1230
cctatctcca tctctgact agccactgag gtgaattggg acgaagaaaa ggaatgtttt 1240
10 gaaagcctca gtaaaagtgt cgtatgttct tattccatcc ggaagcagta catatctgag 1250
gagtgcaccc tctcaggcca gcagagtga gtgcttggtt ccatttccaa ctctgggaag 1260
tggactgttg aacacattgt ctataaagcc ttgogctcac acattctgct tctaaacat 1270
ttcacagaag atggaaatat cctgcagctt gctaacctgc ctgactctata caaagtcttt 1280
gagaggtgtt aaatatggtt atttatgcac tgtgggatgt gttctctctt ctctgtatto 1290
15 cgatacaaa tggttgtatca aagtgtgata taaaaagtgt accaacataa gtgttggtag 1300
cacttaagac ttatacttgc cttctgatag tattccttta tacacagtgg attgattata 1310
aataaataga tgtgtcttaa cata 1324

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hPMS2-134 (human) (SEQ ID NO:15)

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20  MERAESSSTE PAKAIKFIDR KSVHQCISGQ VVLSLSTAVK ELVENSIDAG ATNIDLKLKD 60
    YGVDLIEVSD NCGVEEENF EGLTLKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
    TISTCHASAK VGT 132

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hPMS2-134 (human cDNA) (SEQ ID NO:16)

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25  cgaggcgggat cgggtgttgc atccatggag cgagctgaga gctcgagtag agaactgtct 60
aagccatcca aacctattga toggaaagca gtccatcaga ttgtctctgg gcaggtggta 120
ctgagctctaa gcactgcggt aaaggagtta gtgaaaaaca gtctggatgc tgggtgccact 180
aatattgata taaaacttaa ggaactatga gtggatctta ttgaagtctc agacaatgga 240
tgtggggtag aagaagaaaa cttcgaagga ttaactctga aacatcacac atctaagatt 300
30  caagagtttg ccgacctaac tcagggtgaa acttttggct ttggggggga agctctgagc 360
tcactttgtg cactgagcga tgtcaccatt tctacctgcc acgcacgggc gaaggttgga 420
acttga 426

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For further information on the background of the invention the following references

may be consulted, each of which is incorporated herein by reference in its entirety:

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The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1: Stable expression of dominant negative MMR genes in hybridoma cells

It has been previously shown by Nicolaides *et al.* (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641) that the expression of a dominant negative allele in an otherwise MMR proficient cell could render these host cells MMR deficient. The creation of MMR deficient cells can lead to the generation of genetic alterations throughout the entire genome of a host organisms offspring, yielding a population of genetically altered offspring or siblings that may produce biochemicals with altered properties. This patent application teaches of the use of dominant negative MMR genes in antibody-producing cells, including but not limited to rodent hybridomas, human hybridomas, chimeric rodent cells producing human immunoglobulin gene products, human cells expressing immunoglobulin genes, mammalian cells producing single chain antibodies, and prokaryotic cells producing mammalian immunoglobulin genes or chimeric immunoglobulin molecules such as those contained within single-chain antibodies. The cell expression systems described above that are used to produce antibodies are well known by those skilled in the art of antibody therapeutics.

To demonstrate the ability to create MMR defective hybridomas using dominant negative alleles of MMR genes, we first transfected a mouse hybridoma cell line that is known to produce and antibody directed against the human IgE protein with an expression vector containing the human PMS2 (cell line referred to as HBPMS2), the previously

published dominant negative PMS2 mutant referred herein as PMS134 (cell line referred to as HB134), or with no insert (cell line referred to as HBvec). The results showed that the PMS134 mutant could indeed exert a robust dominant negative effect, resulting in biochemical and genetic manifestations of MMR deficiency. Unexpectedly was the finding that the full length PMS2 also resulted in a lower MMR activity while no effect was seen in cells containing the empty vector. A brief description of the methods is provided below.

The MMR proficient mouse H36 hybridoma cell line was transfected with various *hPMS2* expression plasmids plus reporter constructs for assessing MMR activity. The MMR genes were cloned into the pEF expression vector, which contains the elongation factor promoter upstream of the cloning site followed by a mammalian polyadenylation signal. This vector also contains the NEO^r gene that allows for selection of cells retaining this plasmid. Briefly, cells were transfected with 1 µg of each vector using polyliposomes following the manufacturer's protocol (Life Technologies). Cells were then selected in 0.5 mg/ml of G418 for 10 days and G418 resistant cells were pooled together to analyze for gene expression. The pEF construct contains an intron that separates the exon 1 of the EF gene from exon 2, which is juxtaposed to the 5' end of the polylinker cloning site. This allows for a rapid reverse transcriptase polymerase chain reaction (RT-PCR) screen for cells expressing the spliced products. At day 17, 100,000 cells were isolated and their RNA extracted using the trizol method as previously described (Nicolaidis N.C., Kinzler, K.W., and Vogelstein, B. (1995) Analysis of the 5' region of PMS2 reveals heterogeneous transcripts and a novel overlapping gene. *Genomics* 29:329-334). RNAs were reverse transcribed using Superscript II (Life Technologies) and PCR amplified using a sense primer located in exon 1 of the EF gene (5'-ttt cgc aac ggg ttt gcc g-3') and an antisense primer (5'-gtt tca gag tta agc ctt cg-3') centered at nt 283 of the published human PMS2 cDNA, which will detect both the full length as well as the PMS134 gene expression. Reactions were carried out using buffers and conditions as previously described (Nicolaidis, N.C., *et al.* (1995) Genomic organization of the human PMS2 gene family. *Genomics* 30:195-206), using the following amplification parameters: 94°C for 30 sec, 52°C for 2 min, 72°C for 2 min, for 30 cycles. Reactions were analyzed on agarose gels. Figure 1 shows a representative example of PMS expression in stably transduced H36 cells.

Expression of the protein encoded by these genes were confirmed via western blot using a polyclonal antibody directed to the first 20 amino acids located in the N-terminus of the protein following the procedures previously described (data not shown) (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641.

EXAMPLE 2: hPMS134 Causes a Defect in MMR Activity and hypermutability in hybridoma cells

A hallmark of MMR deficiency is the generation of unstable microsatellite repeats in the genome of host cells. This phenotype is referred to as microsatellite instability (MI) (Modrich, P. (1994) Mismatch repair, genetic stability, and cancer *Science* 266:1959-1960; Palombo, F., *et al.* (1994) Mismatch repair and cancer *Nature* 36:417). MI consists of deletions and/or insertions within repetitive mono-, di- and/or tri nucleotide repetitive sequences throughout the entire genome of a host cell. Extensive genetic analysis eukaryotic cells have found that the only biochemical defect that is capable of producing MI is defective MMR (Strand, M., *et al.* (1993) Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair *Nature* 365:274-276; Perucho, M. (1996) Cancer of the microsatellite mutator phenotype. *Biol Chem.* 377:675-684; Eshleman J.R., and Markowitz, S.D. (1996) Mismatch repair defects in human carcinogenesis. *Hum. Mol. Genet.* 5:1489-494). In light of this unique feature that defective MMR has on promoting MI, it is now used as a biochemical marker to survey for lack of MMR activity within host cells (Perucho, M. (1996) Cancer of the microsatellite mutator phenotype. *Biol Chem.* 377:675-684; Eshleman J.R., and Markowitz, S.D. (1996) Mismatch repair defects in human carcinogenesis. *Hum. Mol. Genet.* 5:1489-494; Liu, T., *et al.* (2000) Microsatellite instability as a predictor of a mutation in a DNA mismatch repair gene in familial colorectal cancer *Genes Chromosomes Cancer* 27:17-25).

A method used to detect MMR deficiency in eukaryotic cells is to employ a reporter gene that has a polynucleotide repeat inserted within the coding region that disrupts its reading frame due to a frame shift. In the case where MMR is defective, the reporter gene will acquire random mutations (i.e. insertions and/or deletions) within the polynucleotide

repeat yielding clones that contain a reporter with an open reading frame. We have employed the use of an MMR-sensitive reporter gene to measure for MMR activity in HBvec, HBPMS2, and HBPMS134 cells. The reporter construct used the pCAR-OF, which contains a hygromycin resistance (HYG) gene plus a β -galactosidase gene containing a 29 bp

5 out-of-frame poly-CA tract at the 5' end of its coding region. The pCAR-OF reporter would not generate β -galactosidase activity unless a frame-restoring mutation (*i.e.*, insertion or deletion) arose following transfection. HBvec, HBPMS2, and HB134 cells were each transfected with pCAR-OF vector in duplicate reactions following the protocol described in Example 1. Cells were selected in 0.5 mg/ml G418 and 0.5mg/ml HYG to select for cells
10 retaining both the MMR effector and the pCAR-OF reporter plasmids. All cultures transfected with the pCAR vector resulted in a similar number of HYG/G418 resistant cells. Cultures were then expanded and tested for β -galactosidase activity *in situ* as well as by biochemical analysis of cell extracts. For *in situ* analysis, 100,000 cells were harvested and fixed in 1% glutaraldehyde, washed in phosphate buffered saline solution and incubated in 1
15 ml of X-gal substrate solution [0.15 M NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, 0.2% X-Gal] in 24 well plates for 2 hours at 37°C. Reactions were stopped in 500 mM sodium bicarbonate solution and transferred to microscope slides for analysis. Three fields of 200 cells each were counted for blue (β -galactosidase positive cells) or white (β -galactosidase negative cells) to assess for MMR inactivation. Table 1 shows the results
20 from these studies. While no β -galactosidase positive cells were observed in HBvec cells, 10% of the cells per field were β -galactosidase positive in HB134 cultures and 2% of the cells per field were β -galactosidase positive in HBPMS2 cultures.

Cell extracts were prepared from the above cultures to measure β -galactosidase using a quantitative biochemical assay as previously described (Nicolaides *et al.* (1998)

25 A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641; Nicolaides, N.C., *et al.* (1992) The Jun family members, c-JUN and JUND, transactivate the human *c-myc* promoter via an Ap1 like element. *J. Biol. Chem.* 267:19665-19672). Briefly, 100,000 cells were collected, centrifuged and resuspended in 200 μ l of 0.25M Tris, pH 8.0. Cells were

lysed by freeze/thawing three times and supernatants collected after microfugation at 14,000 rpms to remove cell debris. Protein content was determined by spectrophotometric analysis at OD²⁸⁰. For biochemical assays, 20 µg of protein was added to buffer containing 45 mM 2-mercaptoethanol, 1mM MgCl₂, 0.1 M NaPO₄ and 0.6 mg/ml Chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim). Reactions were incubated for 1 hour, terminated by the addition of 0.5 M Na₂CO₃, and analyzed by spectrophotometry at 576 nm. H36 cell lysates were used to subtract out background. Figure 2 shows the β-galactosidase activity in extracts from the various cell lines. As shown, the HB134 cells produced the highest amount of β-galactosidase, while no activity was found in the HBvec cells containing the pCAR-OF. These data demonstrate the ability to generate MMR defective hybridoma cells using dominant negative MMR gene alleles.

Table 1. β-galactosidase expression of HBvec, HBPMS2 and HB134 cells transfected with pCAR-OF reporter vectors. Cells were transfected with the pCAR-OF β-galactosidase reporter plasmid. Transfected cells were selected in hygromycin and G418, expanded and stained with X-gal solution to measure for β-galactosidase activity (blue colored cells). 3 fields of 200 cells each were analyzed by microscopy. The results below represent the mean +/- standard deviation of these experiments.

Table 1.

| CELL LINE | # BLUE CELLS |
|-----------|--------------|
| HBvec | 0 +/- 0 |
| HPMS2 | 4 +/- 1 |
| HB134 | 20 +/- 3 |

EXAMPLE 3: Screening strategy to identify hybridoma clones producing antibodies with higher binding affinities and/or increased immunoglobulin production.

An application of the methods presented within this document is the use of MMR deficient hybridomas or other immunoglobulin producing cells to create genetic alterations within an immunoglobulin gene that will yield antibodies with altered biochemical properties.

An illustration of this application is demonstrated within this example whereby the HB134 hybridoma (see Example 1), which is a MMR-defective cell line that produces an anti-human immunoglobulin type E (hIgE) MAb, is grown for 20 generations and clones are isolated in 96-well plates and screened for hIgE binding. Figure 3 outlines the screening procedure to identify clones that produce high affinity MAbs, which is presumed to be due to an alteration within the light or heavy chain variable region of the protein. The assay employs the use of a plate Enzyme Linked Immunosorbant Assay (ELISA) to screen for clones that produce high-affinity MAbs. 96-well plates containing single cells from HBvec or HB134 pools are grown for 9 days in growth medium (RPMI 1640 plus 10% fetal bovine serum) plus 0.5 mg/ml G418 to ensure clones retain the expression vector. After 9 days, plates are screened using an hIgE plate ELISA, whereby a 96 well plate is coated with 50µls of a 1µg/ml hIgE solution for 4 hours at 4°C. Plates are washed 3 times in calcium and magnesium free phosphate buffered saline solution (PBS^{-/-}) and blocked in 100µls of PBS^{-/-} with 5% dry milk for 1 hour at room temperature. Wells are rinsed and incubated with 100 µls of a PBS solution containing a 1:5 dilution of conditioned medium from each cell clone for 2 hours. Plates are then washed 3 times with PBS^{-/-} and incubated for 1 hour at room temperature with 50 µls of a PBS^{-/-} solution containing 1:3000 dilution of a sheep anti-mouse horse radish peroxidase (HRP) conjugated

secondary antibody. Plates are then washed 3 times with PBS⁺ and incubated with 50 μ ls of TMB-HRP substrate (BioRad) for 15 minutes at room temperature to detect amount of antibody produced by each clone. Reactions are stopped by adding 50 μ ls of 500mM sodium bicarbonate and analyzed by OD at 415nm using a BioRad plate reader. Clones exhibiting an enhanced signal over background cells (H36 control cells) are then isolated and expanded into 10 ml cultures for additional characterization and confirmation of ELISA data in triplicate experiments. ELISAs are also performed on conditioned (CM) from the same clones to measure total Ig production within the conditioned medium of each well. Clones that produce an increased ELISA signal and have increased antibody levels are then further analyzed for variants that over-express and/or over-secrete antibodies as described in Example 4. Analysis of five 96-well plates each from HBvec or HB134 cells have found that a significant number of clones with a higher Optical Density (OD) value is observed in the MMR-defective HB134 cells as compared to the HBvec controls. Figure 4 shows a representative example of HB134 clones producing antibodies that bind to specific antigen (in this case IgE) with a higher affinity. Figure 4 provides raw data from the analysis of 96 wells of HBvec (left graph) or HB134 (right graph) which shows 2 clones from the HB134 plate to have a higher OD reading due to 1) genetic alteration of the antibody variable domain that leads to an increased binding to IgE antigen, or 2) genetic alteration of a cell host that leads to over-production/secretion of the antibody molecule. Anti-Ig ELISA found that the two clones, shown in figure 4 have Ig levels within their CM similar to the surrounding wells exhibiting lower OD values. These data suggest that a genetic alteration occurred within the antigen binding domain of the antibody which in turn allows for higher binding to antigen.

Clones that produced higher OD values as determined by ELISA were further analyzed at the genetic level to confirm that mutations within the light or heavy chain variable region have occurred that lead to a higher binding affinity hence yielding to a stronger ELISA signal. Briefly, 100,000 cells are harvested and extracted for RNA using the Triazol method as described above. RNAs are reverse transcribed using Superscript II as suggested by the manufacturer (Life Technology) and PCR amplified for the antigen binding sites contained within the variable light and heavy chains. Because of the heterogeneous nature of these

genes, the following degenerate primers are used to amplify light and heavy chain alleles from the parent H36 strain.

Light chain sense: 5'-GGA TTT TCA GGT GCA GAT TTT CAG-3' (SEQ ID NO:1)

5

Light chain antisense: 5'-ACT GGA TGG TGG GAA GAT GGA-3' (SEQ ID NO:2)

Heavy chain sense: 5'-A(G/T) GTN (A/C)AG CTN CAG (C/G)AG TC-3' (SEQ ID NO:3)

10 Heavy chain antisense: 5'-TNC CTT G(A/G)C CCC AGT A(G/A)(A/T)C-3' (SEQ ID NO:4)

PCR reactions using degenerate oligonucleotides are carried out at 94°C for 30 sec, 52°C for 1 min, and 72°C for 1 min for 35 cycles. Products are analyzed on agarose gels. Products of the expected molecular weights are purified from the gels by Gene Clean (Bio 15 101), cloned into T-tailed vectors, and sequenced to identify the wild type sequence of the variable light and heavy chains. Once the wild type sequence has been determined, non-degenerate primers were made for RT-PCR amplification of positive HB134 clones. Both the light and heavy chains were amplified, gel purified and sequenced using the corresponding sense and antisense primers. The sequencing of RT-PCR products gives representative 20 sequence data of the endogenous immunoglobulin gene and not due to PCR induced mutations. Sequences from clones were then compared to the wild type sequence for sequence comparison. An example of the ability to create *in vivo* mutations within an immunoglobulin light or heavy chain is shown in figure 5, where HB134 clone92 was identified by ELISA to have an increased signal for hIgE. The light chain was amplified 25 using specific sense and antisense primers. The light chain was RT-PCR amplified and the resulting product was purified and analyzed on an automated ABI377 sequencer. As shown in clone A, a residue -4 upstream of the CDR region 3 had a genetic change from ACT to TCT, which results in a Thr to Ser change within the framework region just preceding the CDR#3. In clone B, a residue -6 upstream of the CDR region had a genetic change from CCC to CTC, 30 which results in a Pro to His change within framework region preceding CDR#2.

The ability to generate random mutations in immunoglobulin genes or chimeric immunoglobulin genes is not limited to hybridomas. Nicolaides et al. (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641) has previously shown the ability to generate hypermutable hamster cells and produce mutations within an endogenous gene. A common method for producing humanized antibodies is to graft CDR sequences from a MAb (produced by immunizing a rodent host) onto a human Ig backbone, and transfection of the chimeric genes into Chinese Hamster Ovary (CHO) cells which in turn produce a functional Ab that is secreted by the CHO cells (Shields, R.L., *et al.* (1995) Anti-IgE monoclonal antibodies that inhibit allergen-specific histamine release. *Int. Arch. Allergy Immunol.* 107:412-413). The methods described within this application are also useful for generating genetic alterations within Ig genes or chimeric Igs transfected within host cells such as rodent cell lines, plants, yeast and prokaryotes (Frigerio L, *et al.* (2000) Assembly, secretion, and vacuolar delivery of a hybrid immunoglobulin in plants. *Plant Physiol.* 123:1483-1494).

These data demonstrate the ability to generate hypermutable hybridomas, or other Ig producing host cells that can be grown and selected, to identify structurally altered immunoglobulins yielding antibodies with enhanced biochemical properties, including but not limited to increased antigen binding affinity. Moreover, hypermutable clones that contain missense mutations within the immunoglobulin gene that result in an amino acid change or changes can be then further characterized for *in vivo* stability, antigen clearance, on-off binding to antigens, etc. Clones can also be further expanded for subsequent rounds of *in vivo* mutations and can be screened using the strategy listed above.

The use of chemical mutagens to produce genetic mutations in cells or whole organisms are limited due to the toxic effects that these agents have on "normal" cells. The use of chemical mutagens such as MNU in MMR defective organisms is much more tolerable yielding to a 10 to 100 fold increase in genetic mutation over MMR deficiency alone (Bignami M, (2000) Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. *Mutat. Res.* 462:71-82). This strategy allows for the use of chemical mutagens to be used in MMR-defective Ab producing cells as a method for increasing

additional mutations within immunoglobulin genes or chimeras that may yield functional Abs with altered biochemical properties such as enhanced binding affinity to antigen, etc.

Example 4: Generation of antibody producing cells with enhanced antibody production

5 Analysis of clones from H36 and HB134 following the screening strategy listed above has identified a significant number of clones that produce enhanced amounts of antibody into the medium. While a subset of these clones gave higher Ig binding data as determined by ELISA as a consequence of mutations within the antigen binding domains contained in the variable regions, others were found to contain "enhanced" antibody production. A summary
10 of the clones producing enhanced amounts of secreted MAb is shown in TABLE 2, where a significant number of clones from HB134 cells were found to produce enhanced Ab production within the conditioned medium as compared to H36 control cells.

TABLE 2. Generation of hybridoma cells producing high levels of antibody. HB134 clones
15 were assayed by ELISA for elevated Ig levels. Analysis of 480 clones showed that a significant number of clones had elevated MAb product levels in their CM. Quantification showed that several of these clones produced greater than 500ngs/ml of MAb due to either enhanced expression and/or secretion as compared to clones from the H36 cell line.

20 **Table 2. Production of MAb in CM from H36 and HB134 clones.**

| Cell Line | % clones > 400 ng/ml | % clones >500 ng/ml |
|-----------|----------------------|---------------------|
| H36 | 1/480 = 0.2% | 0/480 = 0% |
| HB134 | 50/480 = 10% | 8/480 = 1.7% |

Cellular analysis of HB134 clones with higher MAb levels within the conditioned medium (CM) were analyzed to determine if the increased production was simply due to genetic alterations at the Ig locus that may lead to over-expression of the polypeptides forming
25 the antibody, or due to enhanced secretion due to a genetic alteration affecting secretory pathway mechanisms. To address this issue, we expanded three HB134 clones that had increased levels of antibody within their CM. 10,000 cells were prepared for western blot

analysis to assay for intracellular steady state Ig protein levels (Figure 6). In addition, H36 cells were used as a standard reference (Lane 2) and a rodent fibroblast (Lane 1) was used as an Ig negative control. Briefly, cells were pelleted by centrifugation and lysed directly in 300 μ l of SDS lysis buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M

5 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes. Lysate proteins were separated by electrophoresis on 4-12% NuPAGE gels (for analysis of Ig heavy chain. Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked at room temperature for 1 hour in Tris-buffered saline (TBS) plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a
10 1:10,000 dilution of sheep anti-mouse horseradish peroxidase conjugated monoclonal antibody in TBS buffer and detected by chemiluminescence using Supersignal substrate (Pierce). Experiments were repeated in duplicates to ensure reproducibility. Figure 6 shows a representative analysis where a subset of clones had enhanced Ig production which accounted for increased Ab production (Lane 5) while others had a similar steady state level as the
15 control sample, yet had higher levels of Ab within the CM. These data suggest a mechanism whereby a subset of HB134 clones contained a genetic alteration that in turn produces elevated secretion of antibody.

The use of chemical mutagens to produce genetic mutations in cells or whole organisms are limited due to the toxic effects that these agents have on "normal" cells. The
20 use of chemical mutagens such as MNU in MMR defective organisms is much more tolerable yielding to a 10 to 100 fold increase in genetic mutation over MMR deficiency alone (Bignami M, (2000) Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. *Mutat. Res.* 462:71-82). This strategy allows for the use of chemical mutagens to be used in MMR-defective Ab producing cells as a method for increasing
25 additional mutations within immunoglobulin genes or chimeras that may yield functional Abs with altered biochemical properties such as enhanced binding affinity to antigen, etc.

Example 5: establishment of genetic stability in hybridoma cells with new output trait.

The initial steps of MMR are dependent on two protein complexes, called MutS α and
30 MutL α (Nicolaidis *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a

Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641). Dominant negative MMR alleles are able to perturb the formation of these complexes with downstream biochemicals involved in the excision and polymerization of nucleotides comprising the "corrected" nucleotides. Examples from this application show the ability of a truncated MMR allele (PMS134) as well as a full length human PMS2 when expressed in a hybridoma cell line is capable of blocking MMR resulting in a hypermutable cell line that gains genetic alterations throughout its entire genome per cell division. Once a cell line is produced that contains genetic alterations within genes encoding for an antibody, a single chain antibody, over expression of immunoglobulin genes and/or enhanced secretion of antibody, it is desirable to restore the genomic integrity of the cell host. This can be achieved by the use of inducible vectors whereby dominant negative MMR genes are cloned into such vectors, introduced into Ab producing cells and the cells are cultured in the presence of inducer molecules and/or conditions. Inducible vectors include but are not limited to chemical regulated promoters such as the steroid inducible MMTV, tetracycline regulated promoters, temperature sensitive MMR gene alleles, and temperature sensitive promoters.

The results described above lead to several conclusions. First, expression of hPMS2 and PMS134 results in an increase in microsatellite instability in hybridoma cells. That this elevated microsatellite instability is due to MMR deficiency was proven by evaluation of extracts from stably transduced cells. The expression of PMS134 results in a polar defect in MMR, which was only observed using heteroduplexes designed to test repair from the 5' direction (no significant defect in repair from the 3' direction was observed in the same extracts) (Nicolaidis *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641). Interestingly, cells deficient in hMLH1 also have a polar defect in MMR, but in this case preferentially affecting repair from the 3' direction (Drummond, J.T., *et al.* (1996) Cisplatin and adriamycin resistance are associated with MutLa and mismatch repair deficiency in an ovarian tumor cell line. *J. Biol. Chem.* 271:9645-19648). It is known from previous studies in both prokaryotes and eukaryotes that the separate enzymatic components mediate repair from the two different directions. Our results, in combination with those of Drummond *et al.* (Shields, R.L., *et al.* (1995) Anti-IgE monoclonal antibodies that inhibit allergen-specific histamine release. *Int.*

Arch Allergy Immunol. 107:412-413), strongly suggest a model in which 5' repair is primarily dependent on hPMS2 while 3' repair is primarily dependent on hMLH1. It is easy to envision how the dimeric complex between PMS2 and MLH1 might set up this directionality. The combined results also demonstrate that a defect in directional MMR is sufficient to produce a MMR defective phenotype and suggests that any MMR gene allele is useful to produce genetically altered hybridoma cells, or a cell line that is producing Ig gene products. Moreover, the use of such MMR alleles will be useful for generating genetically altered Ig polypeptides with altered biochemical properties as well as cell hosts that produce enhanced amounts of antibody molecules.

Another method that is taught in this application is that ANY method used to block MMR can be performed to generate hypermutability in an antibody-producing cell that can lead to genetically altered antibodies with enhanced biochemical features such as but not limited to increased antigen binding, enhanced pharmacokinetic profiles, etc. These processes can also to be used to generate antibody producer cells that have increased Ig expression as shown in Example 4, figure 6 and/or increased antibody secretion as shown in Table 2.

In addition, we demonstrate the utility of blocking MMR in antibody producing cells to increase genetic alterations within Ig genes that may lead to altered biochemical features such as, but not limited to, increased antigen binding affinities (Figure 5A and 5B). The blockade of MMR in such cells can be through the use of dominant negative MMR gene alleles from any species including bacteria, yeast, protozoa, insects, rodents, primates, mammalian cells, and man. Blockade of MMR can also be generated through the use of antisense RNA or deoxynucleotides directed to any of the genes involved in the MMR biochemical pathway. Blockade of MMR can be through the use of polypeptides that interfere with subunits of the MMR complex including but not limited to antibodies. Finally, the blockade of MMR may be through the use chemicals such as but not limited to nonhydrolyzable ATP analogs, which have been shown to block MMR (Galio, L, *et al.* (1999) ATP hydrolysis-dependent formation of a dynamic ternary nucleoprotein complex with MutS and MutL. *Nucl. Acids Res.* 27:2325-23231).